FLUORESCENCE BASED INTERACTION STUDIES OF NUCLEIC ACIDS (DNA & RNA) WITH HYDROXAMIC ACIDS

INTRODUCTION

Fluorescence is the molecular absorption of light energy at one wavelength and its nearly instantaneous re-emission at another wavelength (usually larger). Fluorescence spectroscopy is an important investigational tool in many areas of analytical science, due to its extremely high sensitivity and selectivity. With many uses across a broad range of chemical, biochemical and medical research, it has become an essential investigational technique allowing detailed, real-time observation of the structure and dynamics of intact biological systems with extremely high resolution. The fluorescence process is governed by three important events, all of which occur on timescales that are separated by several orders of magnitude. Excitation of a susceptible molecule by an incoming photon happens in femtoseconds (10^{15} seconds), while vibrational relaxation of excited state electrons to the lowest energy level is much slower and can be measured in picoseconds (10^{12} seconds). The final process, emission of a longer wavelength photon and return of the molecule to the ground state, occurs in the relatively long time period of nanoseconds (10^{9} seconds). Although the entire molecular fluorescence lifetime, from excitation to emission, is measured in only billionths of a second, the phenomenon is a stunning manifestation of the interaction between light and matter that forms the basis for the expansive fields of steady state and time-resolved fluorescence spectroscopy and microscopy. Because of the tremendously sensitive emission profiles, spatial resolution, and high specificity of fluorescence investigation, this technique has become an important tool in genetics and cell biology.

The principle of this technique is based on the fact that the specimen is illuminated with light of a specific wavelength which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical

components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapour lamp), the excitation filter, the dichoric mirror (or dichoric beamsplitter), and the emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.

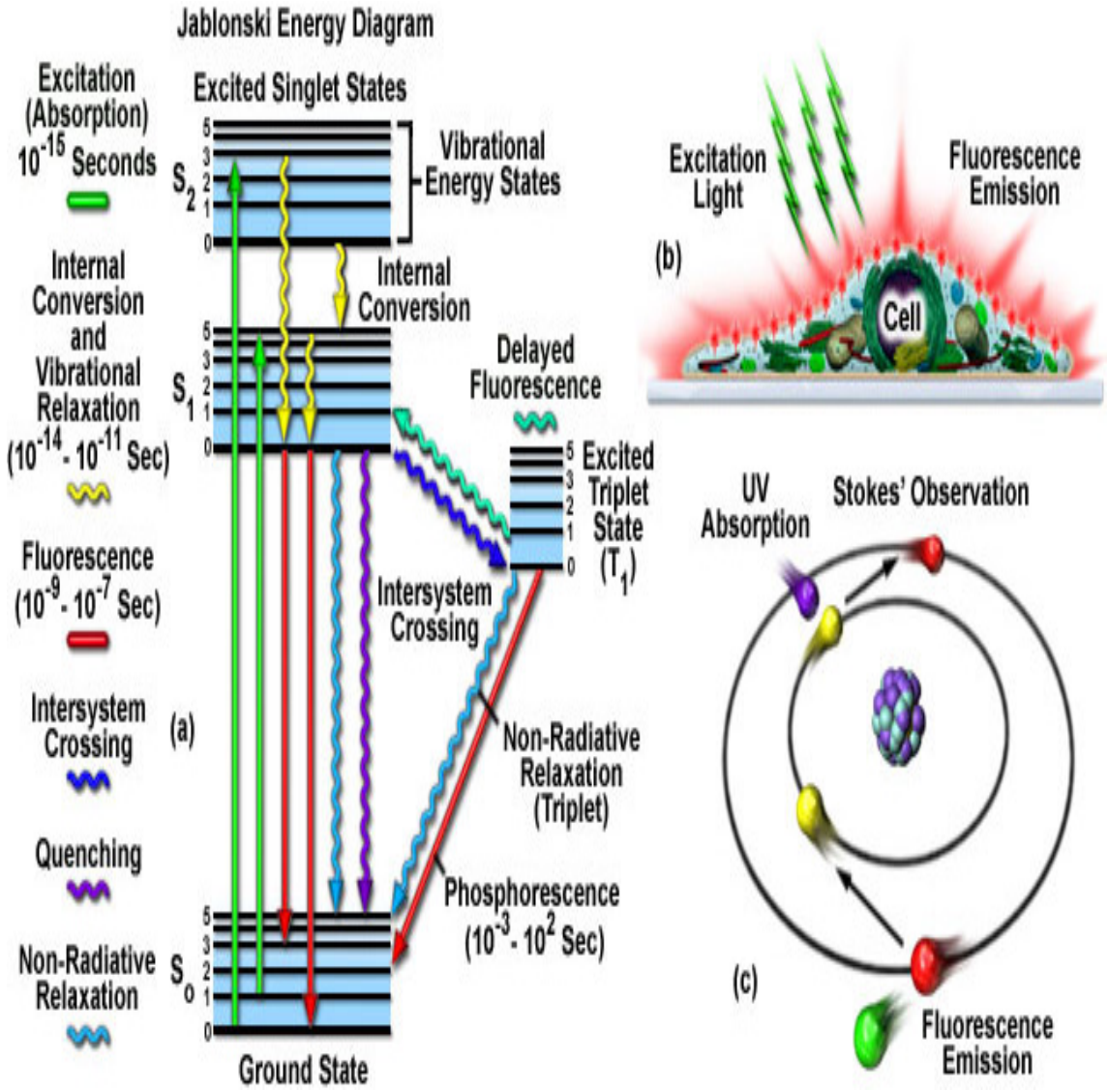


FIG. FUNDAMENTAL CONCEPTS UNDERPINNING FLUORESCENCE MICROSCOPY

Interaction between small molecules and nucleic acids has been an active area that has received considerable attention in the past several years [1-4]. The impetus for these studies has been the

desire to understand the molecular basis of drug action. Extensive studies have led to the discovery of a large number of lead compounds that range from natural products to purely synthetic designs that can bind in a variety of surprising ways to DNA modulating various biological activities. These molecules serve as lead compounds in studies of protein–nucleic acid recognition, provide site specific reagents for molecular biology and serve as potential guides for the rational design of new therapeutic agents. Intercalation and groove binding represent two major noncovalent binding modes of small molecules to DNA and knowledge on the molecular aspects of these binding modes have significantly advanced the development of more efficacious anticancer agents.

In contrast to DNA, over a period of time very little attention has been paid to the recognition of RNA by small molecules. This has been partly due to the complex structural diversity of the RNAs in comparison to DNA and the relatively scanty high-resolution structural information available in respect of RNA molecules. Nevertheless, since the knowledge that many serious diseases are caused by RNA viruses and particularily after the emergence of RNA viruses like HIV and Hepatitis C, there has been growing interest in the development of RNA binding antiviral compounds and RNA based anticancer agents [5–9]. A rational design of such RNA based therapeutics molecules however would essentially require a detailed knowledge of the structural aspects of RNA on one hand and the molecular nature of the mode, mechanism and specificity of binding of small molecules to various conformations of RNA on the other hand. In the past several years significant advancement has taken place in the structural evaluation of various RNAs [10, 11]. Although all cellular RNAs have single polynucleotide chain, they are highly versatile molecules that can fold into a multitude of secondary structures and conformations. These complex structural motifs could be potential binding pockets for specific drug recognition sites and it would be interesting to take advantage of these promising recognition capabilities of RNAs to develop new RNA binders as modulators of cellular functions [12–14].

DNA plays a major role in biological systems because it carries most of the heritage information and instructs the biological synthesis of proteins and enzymes through the process of replication and transcription of genetic information [15]. It is quite often the primary intracellular target of anticancer drugs within an organism , therefore, there is growing interest in exploring the binding of small molecules with DNA for the rational design and construction of new and more targeted to

DNA [16]. Small molecule can interact with DNA through the following three non-covalent modes:

(i) Intercalation between the base pairs;
(ii) Groove binding, interactions with the DNA groove; and
(iii) Electrostatic interaction (electrostatic attractions with the anionic sugar-phosphate backbone of DNA).

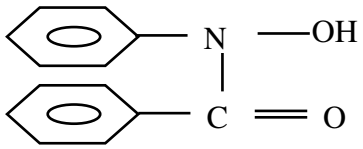
Intercalative binding and groove binding are related to the grooves in the DNA double helix, while the electrostatic binding takes place out of the groove [17].

In general, small molecules are stabilized on groove binding and intercalation with DNA through a series of associative interactions such as π-stacking, hydrogen bond, attractive Vander Waals and hydrophobic interaction [18]. The intercalative binding is stronger than other two binding modes because the surface of intercalative molecule is inserted into base pairs of DNA, it may lead to the occurrence of DNA damage, even further affecting the expression of genetic information replication.

Hydroxamic acids RCONR'OH have been known since 1869 with the discovery of oxalohydroxamic acid by Lossen [19]. These weak acids, whether naturally occurring or synthetic, constitute one of the most important families of organic bioligands. They are widely distributed in all tissues of plants as gowth factors and vitamins in microbes [20] and also a key pharmacophore in a number of important chemotherapeutic agents. Pharmacological applications of these compounds include tumour inhibitors, antimicrobial agents, antituberculous, antilukemic agents [21]. Several of hydroxamic acid derivatives have entered into human clinical trials for the treatment of cancer and arthritis [22]. They are widely known metal chelators especially iron (III), aluminium (III) and vanadium (IV). Due to their ability to chelate high density metal ions they can be used as possible imaging agents [23] .

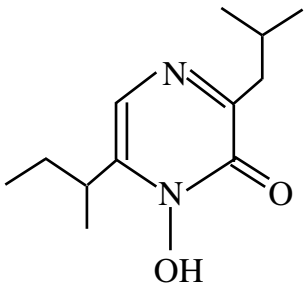
In general, hydroxamic acids are the N-acyl/aryl derivatives of hydroxylamine as shown in figure (I). Bamberger in 1919 [24] laid the foundation of N-arylhydroxamic acids by synthesizing the parent compound of this series, N-phenylbenzohydroxamic acid, trivially named as PBHA, (III).

After the recognition of its analytical potentialities by Shome [25], a large number of analogs of PBHA have been synthesized and reported [26-31].



(I)

Hydroxamic acids are wide-spread in the tissues of plants, in metabolites of bacteria and fungi including complex compounds. The first naturally occurring hydroxamic acid is aspergillic acid (II) reported from fungi and actinomycetes.



(II)

A BRIEF REVIEW OF THE WORK ALREADY DONE IN THE FIELD AND SIGNIFICANT CONTRIBUTION IN THE FIELD OF PROPOSED WORK

N- Arylhydroxamic acids show biological activity as reported by this laboratory. Various QSAR parameters of these molecules have been determined and reported [32, 33]. The knowledge of

binding interactions of DNA with N-arylhydroxamic acids is totally lacking. Therefore present investigation is undertaken.

OBJECTIVE

In our laboratory, we have focused our attention to drugs and their effect on target cellular DNA and RNA to understand the mechanism of action at the molecular level, and also to investigate the effect of drugs as anticancer agents and antitumor agent. Therefore in the present investigation, the interaction of hydroxamic acids with nucleic acids will investigated following the fluorescence techniques.

(i) Fluorescence Spectroscopic method

(ii) Fluorescence Microscopy

PROPOSED METHODOLOGY

The work will be distributed in the following chapters.

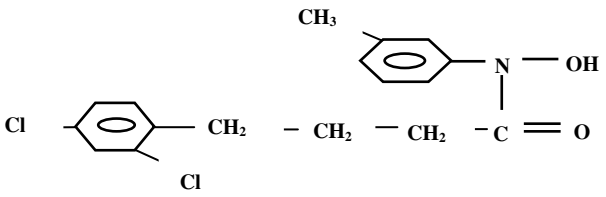
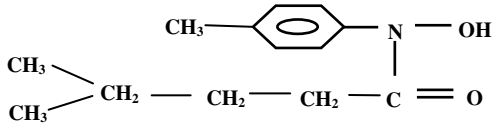
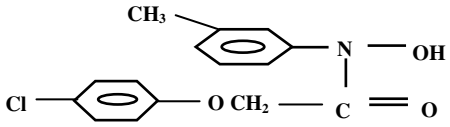
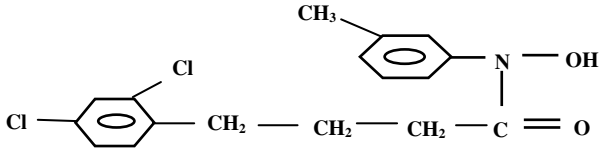
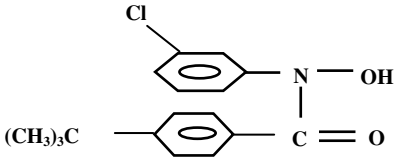
[1] INTRODUCTION

In this chapter, introduction of hydroxamic acids and nucleic acids will be described with recent literature survey data.

[2] SYNTHESIS OF HYDROXAMIC ACIDS

N-Arylhydroxamic acids and their derivatives will be synthesized in the laboratory following the steps reported in literature [34, 35]. These will be further purified by crystallizing thrice with benzene before use.The purity of the products will be ascertained by determining their melting points and spectral analyses (UV and IR).

Following hydroxamic acids will be applied for the present investigation,

S.NO.	HYDROXAMIC ACID	MOLECULAR STURCTURE
1.	N-m Tolyl 2,4 dichlorophenoxyglutyro	
2.	N-p-Tolyl-isovlero	
3.	N-p-Tolyl-p-chloro pheoxy aceto	
4.	N-Phenyl 2 ,4-dichloro phenoxy butyro	
5.	N-m-Chloro-phenyl-p-tert-butyl-benzo	

[3] DETERMINATION OF BINDING PARAMETERS

Following methods will be used to determine the DNA-binding parameters:-

(I) BY UV-VISIBLE ABSORPTION

The intrinsic binding constant of hydroxamic acids with DNA/RNA through UV absorption will be calculated from equation [1],

$$[\text{Ct-DNA}]/(\varepsilon_a - \varepsilon_f) = [\text{Ct-DNA}]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \quad \textbf{[1]}$$

where,

Ct- DNA = base pair concentration

ϵ_b = apparent absorption coefficient for bounded DNA.

ϵ_f = apparent absorption coefficient for free DNA.

K_b = binding constant

(II) VISCOSITY MEASUREMENT

DNA and RNA viscosity is sensitive to their length change, therefore its measurement upon addition of a compound is often concern as the least ambiguous method to clarify the interaction mode of a compound with both and provides reliable evidence for the intercalative binding mode [36-40]. Viscosity measurements will be carried out with Ct-DNA and RNA solutions as a function of ligands concentration.

This experiment is an effective tool to determine the binding mode of small molecules with DNA/RNA. The relative specific viscosity will be then obtained by Eq. **[2]**,

$$\eta / \eta_0 = (t-t_0)/ (ctDNA-t_0) \quad \textbf{[2]}$$

where,

t_0 = flow time of buffer,

t_{DNA} = flow time of buffer containing DNA /RNA,

t = flow time of buffer containing DNA/RNA after the addition of hydroxamic acid,

η_0 = specific viscosity contributions of DNA/ RNA in the absence of complex,

η = specific viscosity contributions of DNA/RNA in the presence of the complex.

(III) BY FLUORESCENCE QUENCHING METHOD

A quantitative estimation of the quenching in terms of the fluorescence quenching constant will be obtained by using the Stern-Volmer equation **[3]**,

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad \textbf{[3]}$$

where,

F_0 = fluorescence intensities of hydroxamic acid in absence,

F = fluorescence intensities of hydroxamic acid in presence of the quencher,

K_{SV} = Stern-Volmer dynamic quenching constant,

[Q] = concentration of quencher.

(IV) COMPETITIVE BINDING WITH ETHIDIUM BROMIDE

Further support for the mode of binding between hydroxamic acids and DNA will be given through the competitive experiment. Here ethidium bromide (EB) can be employed in the examination of the reaction, as EB presumably binds initially to DNA by intercalation.The increase or decrease in the fluorescence intensity will provide the information regarding the nature of intercalation.

(V) BY MELTING STUDIES

The double-helical structure of DNA is remarkably stable due to hydrogen bonding and base stacking interaction. Upon increasing the temperature the dissociation of helix to single strand increases. The melting temperature T_m, which defines as the temperature where half of total base pair is unbounded, will be determined as transition midpoint of melting curve [41]. The change in T_m gives the information of interaction of hydroxamic acids to DNA/RNA.

(VI) THERMODYNAMIC PARAMETERS

The acting forces between small molecules and DNA include, hydrogen bond, Van der Waals forces, hydrophobic interaction and electrostatic forces. According to Ross, binding forces are estimated by measuring ΔH and ΔS, following the Van't Hoff equation,

$$\mathbf{Log\ K = \Delta H/2.303RT + \Delta S/2.303R} \qquad \mathbf{[4]}$$

$$\mathbf{\Delta G = \Delta H - T\ \Delta S} \qquad \mathbf{[5]}$$

(VII) NUCLEIC ACID CLEAVAGE METHOD

Electrophoresis is a useful method for separating and detecting molecules such as protein and nucleic acid such as DNA and RNA. The nucleic acid cleavage ability of the compound will be investigated by gel electrophoresis technique. The results will be analyses by visualizing the gel with UV transilluminator [42, 43].

(VII) FLUORESCENCE MICROSCOPY

Flourescence microscope is basically a light microscope that uses the fluorescence light eminating from fluorescent dye within the sample for imaging. Flourescence microscopy is an unique way of using a microscope to discover facts about specimen that often are not known by standard bright field microscopy. Here, specimen is self-illuminated by internal light, so bright objects are seen more easily. It can be used to find out the effect of the molecules on the higher-order structure of DNA through single-molecule observed in solution.

The diffusion constant, D for each nucleic acid molecule will be calculated [44-46] by using the following eq. **[6]**.

$$(\mathbf{R(t) - R(0)^2}) = \mathbf{4Dt + At^2} \qquad [\mathbf{6}]$$

where,

R(t) = (R_x, R_y) is the position of the centre of mass for a DNAand RNA,

$(R(t) - R(0)^2)$ = Mean square displacement,

A = Numerical constant related to convective flow.

The effective hydrodynamic radius of a single nucleic acid molecule will then be obtained from the Stokes-Einstein relationship [47, 48] given in eq. **[7].**

$$\mathbf{R_H = \frac{k_B T}{6\pi \eta_s D}} \qquad [7]$$

where,

k_B = Boltzmann constant,

η_s = Viscosity of the solvent.

EXPECTED OUTCOME OF THE PROPOSED WORK

Generally, organic molecules are less soluble in water but are more lipophilic and more permeable through membrane in their unionized form. It has been reported that biological activity of hydroxamic acids may be due to the similar structure present in proteins [49]. Since, hydroxamic acids contain the smallest unit –C=O.NH, that can bind to DNA helix, therefore knowledge of favoured binding site and conformational aspects are important to understand the role played by hydroxamic acids in biological processes and tumor/cancer drug designing, as IC_{50} values and QSAR analyses of some of the derivatives are reported from this laboratory [50-53].

This piece of research work will be a small step for fulfilling the quest of knowledge regarding the DNA and RNA binding capacity and various related parameters of hydroxamic acids. It will be a continuation of the work done and reported from this laboratory on "QSAR and Drug Dsigning for Anti-tumor / Anti-cancer Activity", in search of better molecule.

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